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Proliferating Dendritic Cell Progenitors in Human Blood

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Summary

CD34$^+$ cells in human cord blood and marrow are known to give rise to dendritic cells (DC), as well as to other myeloid lineages. CD34$^+$ cells are rare in adult blood, however, making it difficult to use CD34$^+$ cells to ascertain if DC progenitors are present in the circulation and if blood can be a starting point to obtain large numbers of these immunostimulatory antigen-presenting cells for clinical studies. A systematic search for DC progenitors was therefore carried out in several contexts. In each case, we looked initially for the distinctive proliferating aggregates that were described previously in mice. In cord blood, it was only necessary to deplete erythroid progenitors, and add granulocyte/macrophage colony-stimulating factor (GM-CSF) together with tumor necrosis factor (TNF), to observe many aggregates and the production of typical DC progeny. In adult blood from patients receiving CSFs after chemotherapy for malignancy, GM-CSF and TNF likewise generated characteristic DCs from HLA-DR negative precursors. However, in adult blood from healthy donors, the above approaches only generated small DC aggregates which then seemed to become monocytes. When interleukin 4 was used to suppress monocyte development (Jansen, J. H., G.-J. H. M. Wientjens, W. E. Fibbe, R. Willemze, and H. C. Kluin-Nelemans. 1989. *J. Exp. Med.* 170:577.), the addition of GM-CSF led to the formation of large proliferating DC aggregates and within 5–7 d, many nonproliferating progeny, about 3–8 million cells per 40 ml of blood. The progeny had a characteristic morphology and surface composition (e.g., abundant HLA-DR and accessory molecules for cell-mediated immunity) and were potent stimulators of quiescent T cells. Therefore, large numbers of DCs can be mobilized by specific cytokines from progenitors in the blood stream. These relatively large numbers of DC progeny should facilitate future studies of their FcεRI and CD4 receptors, and their use in stimulating T cell–mediated resistance to viruses and tumors.

Dendritic cells (DC)[2] provide an effective pathway for presenting antigens to T cells in situ, both self-antigens during T cell development and foreign antigens during immunity (for a review see reference 1). As such, the delineation of developmental pathways for DCs themselves is of some importance. By identifying the sites in which DC progenitors are found, as well as the requisite cytokines for their proliferation and maturation, one would have much better access to a cell type that mediates clonal deletion of autoreactive T cells in the thymus (2) as well as clonal sensitization of peripheral T cells (3–6).

Two features are well described for the DC developmental pathway: DCs can originate from bone marrow progenitors (7–10), and both the proliferation and maturation of DCs are enhanced by the cytokine GM-CSF (11–21). Early detailed descriptions of the formation of DCs from proliferating progenitors were obtained using mouse blood (11) and shortly thereafter, mouse marrow (12). It was noted that an MHC class II negative precursor could be driven to form distinctive, proliferating aggregates which in turn gave rise to typ-

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[2] Abbreviation used in this paper: DC, dendritic cell.

ical nonproliferating DC progeny. The latter exhibit a tetrad of features: irregular cell shape and motility, abundance of surface molecules that are involved in effective antigen presentation, potent stimulation of quiescent T cells in the MLR (11, 12) and mycobacterial response (22), and homing to the T cell areas of lymphoid tissues in situ.

Prior reports have also defined DC precursors in humans, primarily within the CD34$^+$ progenitor pool in cord blood (14, 15) and adult marrow (16, 23). However, the applicability of this information has been somewhat limited by the need to start with the scarce CD34$^+$ subset. To date, the reported yields of progeny also are small if one is aspiring to use DCs to manipulate the immune response in autologous human T cells. Given the capacity of DCs to elicit strong antigen-specific helper and killer T cell responses (1), one would like to identify accessible sites containing human DC progenitors as well as pathways for their proliferative expansion.

Since blood is the most accessible tissue for clinical studies, we set out to extend the findings that were reported in mouse blood to humans. However, when we tried to induce DC growth by adding GM-CSF to human blood, we identified actively proliferating DC aggregates only infrequently. Rather than conclude that DC progenitors were present in mouse blood but absent from human blood, we performed a stepwise analysis of the criteria and progenitor populations that exist in human blood in different situations. As is described here, the parameters that were productive with mouse blood (11) were indeed applicable to humans as long as one began with neonatal cord blood from adults who were receiving CSF replacement after chemotherapy. Knowing that the criteria could be extended from mice to humans, we then returned to normal blood from healthy adults. Conditions for the generation of large numbers of typical DCs from aggregates of proliferating progenitors were identified. A combination of GM-CSF and IL-4 reproducibly provides about 3–8 million potent DCs from a 40-ml blood sample.

Materials and Methods

Culture Medium. We used RPMI 1640 supplemented with 200 mM L-glutamine, 50 μM 2-ME, 20 μg/ml gentamicin, and either 5–10% FCS (56°C for 0.5 h; Seromed-Biochrom KG, Berlin, Germany) or, in some experiments with 5% cord blood serum.

Recombinant Human Cytokines. GM-CSF (3.1×10^6 U/mg) was kindly provided by Dr. E. Liehl (Sandoz Research Institute, Vienna, Austria); TNF-α (6×10^7 U/mg) by Dr. G. R. Adolf (Boehringer Institut für Arzneimittelforschung, Vienna, Austria); and IL-1α (3×10^8 U [D10 assay]/mg) by Dr. P. Lomedico (Hoffmann La Roche Inc., Nutley, NJ). IL-4 was commercially obtained material (10^7 U/mg) (Genzyme Corp., Cambridge, MA) or supernatant from IL-4 gene-transfected COS cells (3×10^5 U/ml), kindly provided by Dr. G. Le Gros (Ciba-Geigy Ltd., Basel, Switzerland). M-CSF (1.9×10^6 U/mg) was a gift of Dr. S. Clark (Genetics Institute, Cambridge, MA). IL-3 and G-CSF were purchased from Genzyme Corp.

mAbs. We used the following mouse mAbs (see reference 24 unless defined here): W6/32, anti-HLA-A,B,C (HB95 from the American Type Culture Collection, [ATCC] Rockville, MD); L243, anti-HLA-DR (Becton Dickinson & Co. [BD], Mountain View, CA); 9.3F10, anti-HLA-DR+DQ (HB180 from ATCC); SPD1, anti-HLA-DQ-related (gift of L. W. Poulter, Royal Free Hospital, London, England); B7/21, anti-HLA-DP (BD); UCHL1, anti-CD45RO (Dako Corp., Glostrup, Denmark); 4G10, anti-CD45RA; 3C10 and LeuM3 (BD), anti-CD14; EBM11, anti-CD68 (Dako); LeuM1, anti-CD15 (BD); LeuM9, anti-CD33 (BD); HPCA-1, anti-CD34 (BD); Leu11b, anti-CD16 (BD); 2A3, anti-CD25 (BD); IV3 (M. Prager, Dartmouth College Medical School, Hanover, NH) and CIKM5 (G. Pilkington, MacCallum Hospital, Melbourne, Australia), anti-FcγRII/CD32; 15-1, anti-FcεRI (J.-P. Kinet, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD [25]); OKT-6, anti-CD1a (Ortho Pharmaceuticals, Raritan, NJ); Leu4 (BD) and OKT-3 (Ortho), anti-CD3; Leu3a+b, anti-CD4 (BD); Leu1, anti-CD5 (BD); Leu2a, anti-CD8 (BD); Leu12, anti-CD19 (BD); Leu16, anti-CD20 (BD); VIB-E3, anti-CD24 (W. Knapp, University of Vienna, Vienna, Austria); G28-5, anti-CD40 (J. A. Ledbetter, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA); TS1/3, anti-leukocyte function-associated antigen 1 (LFA-1)/CD11a and CLB54, anti-CD18 (both from S. T. Pals, Free University, Amsterdam, The Netherlands); LeuM5, anti-CD11c (BD); 7F7, anti-intercellular adhesion molecule 1 (ICAM-1)/CD54 (M. P. Dierich, University of Innsbruck, Innsbruck, Austria); AICD58, anti-LFA-3/CD58 (Immunotech, Marseille, France); BB1, anti-B7/BB1/CD80 (E. A. Clark, University of Washington, Seattle, WA); Lag, anti-Birbeck-granule-associated (M. Kashihara-Sawami, Kyoto University, Kyoto, Japan [26]); VIE-G4, antiglycophorin (O. Majdic, University of Vienna); and Ki-67, proliferation-associated antigen (Dako [27]).

Culture of DC from Cord Blood. Cord blood was collected according to institutional guidelines during normal full-term deliveries. PBMC were isolated by flotation on Lymphoprep (Nycomed, Oslo, Norway), washed, incubated once in saturating concentrations of antiglycophorin mAb anti-HLA-DR and anti-CD3, washed, panned (10 min on ice, then 20 min at room temperature) twice onto bacterial petri dishes coated with goat anti-mouse Ig (H+L) Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). The nonadherent fractions were then plated in 24-well dishes (Costar Corp., Cambridge, MA) and cultured as described in detail in Results.

Culture of DCs from the Blood of Cancer Patients. Peripheral blood was obtained with the informed consent of cancer patients in complete remission during hematopoietic recovery after high-dose consolidation chemotherapy and subcutaneous daily administration of G-CSF (300 μg human rG-CSF [Neupogen; Hoffmann-LaRoche, Basel, Switzerland] to 15 patients with leukemias/lymphomas, and to two patients with solid tumors, or GM-CSF (400 μg human rGM-CSF [Leukomax; Sandoz, Basel, Switzerland] to one patient with leukemia and two patients with solid tumors. PBMC were prepared by sedimentation in Lymphoprep, coated with anti-HLA-DR plus anti-CD3 mAbs, washed, and panned twice as described above. Nonadherent, depleted fractions were then processed according to the protocol described in detail in Results.

Culture of DC from the Blood of Healthy Adults. PBMC were obtained from either 40–100 ml heparinized fresh whole blood or leukocyte-enriched buffy coats (28), and processed as described in detail in Results.

Phenotypic Analysis. We performed phenotypic analysis exactly as described previously (29) by immunolabeling and flow cytometry analysis, and by immunoperoxidase/immunofluorescence on cells cytospun or attached by poly-L-lysine to glass slides.

T Cell Stimulation Assays. Allogeneic primary MLR and oxidative mitogenesis were performed exactly as described (29).

Results

We systematically evaluated three different situations to generate DCs from proliferating progenitors in blood. Our goals were to define requisite criteria and cytokines for proliferating DCs, but at the same time to avoid the need to enrich for $CD34^+$ progenitor populations which are so few in number.

Cord Blood Mononuclear Cells as a Source for DC Progenitors. We began with cord blood, since a prior report had shown that $0.5–10^6$ enriched (>95%) $CD34^+$ cord blood cells could give rise to $1–2.5 \times 10^7$ DCs if cultured for 14 d in a combination of GM-CSF and TNF (14). A limitation to this previous protocol was that cord blood only contains 0.9–2.6% $CD34^+$ cells (30). Therefore, we assessed a prior technique with adult mouse blood (11) in which unfractionated cells or MHC class II negative cells, were cultured in GM-CSF. We found that the varying, yet substantial percentage of nucleated erythroid cells in human cord blood was toxic and that these could be removed by panning with anti-glycophorin A mAb. We began, then with erythroid-depleted cord blood cells with a low buoyant density (<1.077 g/ml) and plated these at $1–2 \times 10^6$/ml in 1 ml of standard medium supplemented with GM-CSF (400–800 U/ml) ± TNF (50 U/ml). The wells were fed every other day by aspirating 0.3 ml medium and adding back 0.5 ml medium with cytokines.

The subsequent events were similar to those described previously with mouse blood. First, small adherent aggregates appeared after 4–7 d (Fig. 1, *A* and *B*). Many of the peripheral cells displayed a veiled or dendritic appearance, and these adhered loosely to a nest of spindle-shaped cells. Nonadherent cells could be removed by careful rinsing in warm medium, but this was not essential. The adherent aggregates enlarged over the next 7–10 d, indicating proliferative activity (Fig. 1 *C*). Typical "veiled" DCs (Fig. 1, *D* and *E*) were then released. These DC aggregates only developed if GM-CSF was added to the medium. TNF although not essential, increased aggregate size and DC yield by 50–100%. It was advantageous to remove the TNF during the last 1–2 d of culture to permit the release of single, mature DCs.

The released DCs were identified by three sets of criteria. First, the cells by inverted phase contrast microscopy showed characteristic thin motile cytoplasmic processes or veils (Fig. 1, *D* and *E*). The typical ultrastructure of DCs was noted by electron microscopy (see below). Only one Langerhans cell granule (Birbeck granule) was found in 100 cell profiles. Second, the DCs had the standard phenotype i.e., HLA-DR rich but negative for markers of other cells, e.g., CD3/14/19/20. Like epidermal Langerhans cells, CD1a was detected but only 1–2% of the cells reacted with an antigen associated with Langerhans cell granules (anti-Lag) (26). It

Figure 1. Development of DCs in liquid culture of cord blood mononuclear cells supplemented with GM-CSF and TNF. After 6 d, small adherent aggregates are visible under the inverted phase contrast microscope (*A*). Higher magnification reveals that they display typical veils at their edges (*white arrows*), and are affixed to adherent spindle-shaped cells (*B*). At day 14, the DC aggregates have become much larger (*C*), and then finally release typical single DCs which display many processes (*D*, bright field), notably characteristic veils (arrow indicates one such veil that appears en face) (*E*, phase contrast). (*A* and *C*) ×25; (*B*) ×100; (*D* and *E*) ×350.

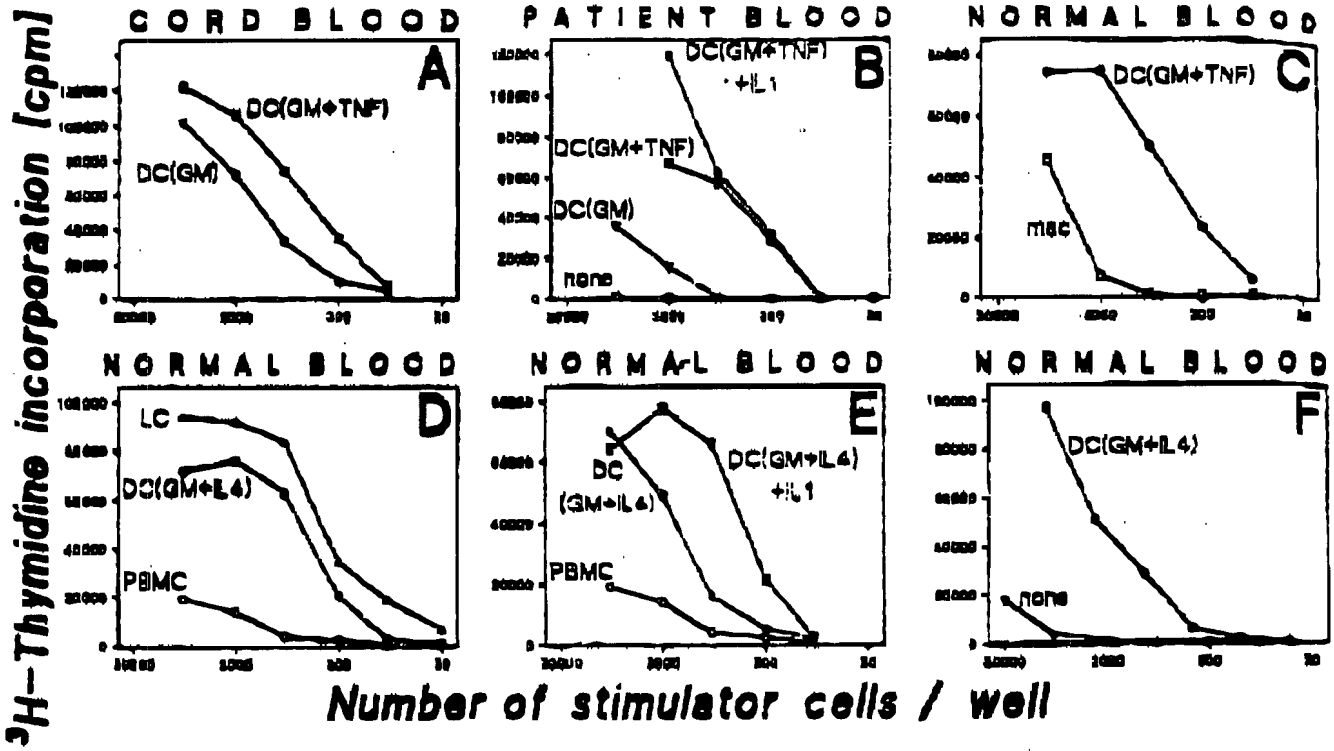

Figure 2. T cell stimulatory function (primary allogeneic MLR) of dendritic cells (*DC*) grown from cord blood with GM-CSF plus TNF-α (*A*), DC grown with GM-CSF plus TNF from the blood of cancer patients after high-dose chemotherapy and G-CSF treatment (*B*), and DC grown from normal peripheral blood with GM-CSF plus TNF-α (*C*), or with GM-CSF plus IL-4 (*D–F*). Responder cells were purified T lymphocytes (2×10^5 in 96 flat bottom wells). Equal numbers of irradiated (3,000 rad, ^{137}Cs) blood DC (●, all panels) as identified by FACS® analysis ($CD1a^+$/HLA-DR$^+$ cells, compare with Fig. 3) were compared both with cultured epidermal Langerhans cells (*LC*) from the same donor in *D* and with poorly stimulating cell populations (whole PBMC in *D* and *E*; adherent macrophages from the same cultures in *C*; and control cultures grown in the absence of cytokines in *F* [Δ]). Note that DC are 10–50-fold stronger than PBMC (*D* and *E*) or macrophages (*C*) and that they are comparable with DC from skin (*D*). In addition, *B* and *E* show the enhancing effect of IL-1 (added during the last 24 h of culture) on the T cell stimulatory capacity of DCs. Without cytokines, no immunostimulatory DCs develop in the cultures (*F*).

is interesting to note that these were in the center of rare residual aggregates. Third, the cord blood–derived DCs were potent stimulators of resting T cells in the primary MLR (Fig. 2 *A*) as well as oxidative mitogenesis (data not shown). The inclusion of TNF in the culture medium increased the immunostimulatory function of the DCs (Fig. 2 *A*).

The above protocol has proven reproducible in 21 standardized experiments and generates $1–5 \times 10^6$ DCs from 40 ml of cord blood at a purity of 20–50% (Table 1). Purity can be increased to >80% by flotation on metrizamide (28) columns. We conclude that (*a*) it is not necessary to enrich for $CD34^+$ precursors to generate typical DCs from cord

Table 1. *DC Progenitors in Human Blood*

Type of blood donor	Enrichment of DC progenitors	Time of culture	DC yields/ 40 ml blood	Percent DC enrichment	Cytokines added
		d		%	
Neonatal, cord blood	Remove glycophorin$^+$ erythroid cells	10–20	$1–5 \times 10^6$	20–50	GM-CSF TNF-α
Adult blood, patients, chemotherapy, and CSF therapy	Remove $CD3^+$ and HLA-DR$^+$ cells	16	$4–8 \times 10^6$	60–80	GM-CSF TNF-α
Adult blood, normal	Bulk PBMC, adherent and loosely adherent	5–7	$3–8 \times 10^6$	40–80	GM-CSF IL-4

blood, and (*b*) the criteria that proved useful in identifying aggregates of proliferating progenitors in mouse blood are also applicable to human cells.

DC Progenitors in the Blood of Cancer Patients during Hematopoietic Recovery from Chemotherapy. We next studied blood mononuclear cells from cancer patients in full remission (leukemias/lymphomas and solid tumors) after high-dose chemotherapy and either G-CSF (17 patients) or GM-CSF (3 patients) treatment. It is known that in the hematopoietic recovery of such patients, progenitors are mobilized into the blood in substantial numbers (0.5–6.0% $CD34^+$ cells) (31, 32). Instead of enriching for $CD34^+$ cells, we simply removed $CD3^+$ and DR^+ cells by panning, and then plated 1–2 × 10^6 cells in 1 ml of medium with 5–10% FCS or 5% cord serum plus 400–800 U/ml GM-CSF. The nonadherent cells were transferred at day 2 (or in some experiments at day 1) and cultured for 16-d feeding every other day.

Growing DC aggregates appeared on day 3–5 and expanded in size until day 11 (data not shown, but compare with Fig. 3). The aggregates developed peripheral veils and initially were loosely attached to a stroma but later were nonadherent. The wells were subcultured, e.g., one well split to two to three wells, when the cell density increased, or if more tightly adherent, smooth, non-DC clusters appeared (contaminating macrophage and granulocyte progenitors). When the DC aggregates became very large (day 12–16), it was easy to dissociate the cells and float the mature DCs on metrizamide columns.

The DCs that developed in this manner had a typical morphology by light and electron microscopy (data not shown, but comparable to Figs. 3 and 6). The phenotype was again MHC class II rich but null for CD3/14/19/20 (data not shown). MLR stimulatory function was potent (Fig. 2 *B*). In contrast to cord blood–derived DCs, CD1a and Lag antigens were not seen (data not shown).

GM-CSF proved essential for DC development. G-CSF, M-CSF, and IL-3 were inactive. Exposure to 3,000 rad of ionizing irradiation blocked DC development. Addition of TNF at 10–50 U/ml usually, though not always, increased DC yields up to twofold, and always improved the function of DCs (Fig. 2 *B*). Human rIL-1 (50 LAF U/ml), when added during the last 24 h in some experiments, further increased function (Fig. 2 *B*).

Starting from 40 ml blood, and using both GM-CSF and TNF, the yield (Table 1) of mature DCs was 4–8 × 10^6 at 16 d with 60–80% purity. This is at least 20 times the yield of mature DCs in fresh normal blood (28, 33).

Proliferating DC Aggregates from Normal Adult Blood. When we applied the above methods to blood from healthy adults, we did observe some small, adherent, veiled aggregates between days 8 and 16. In all 20 experiments, the aggregates then deteriorated and did not enlarge, leaving behind nonviable cells or less often, a few macrophages. Because a stromal monolayer was not evident in the cultures, we next omitted the panning step with anti-CD3 and HLA-DR in case the panning antibodies removed required accessory cells. We simply plated 10^6 bulk mononuclear cells in 1 ml of medium with GM-CSF (800 U/ml) and TNF (50 U/ml), and after

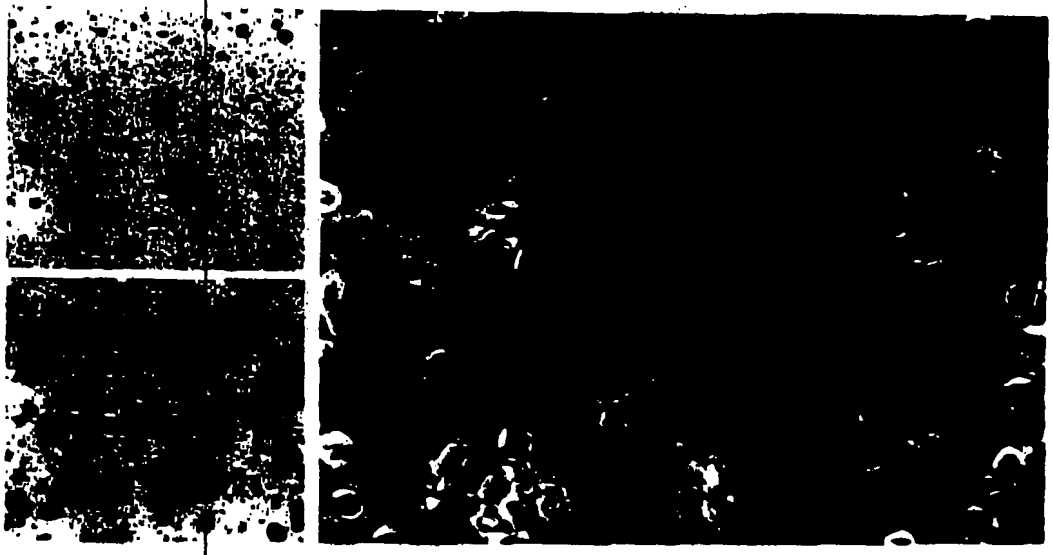

Figure 3. Development of DCs in liquid cultures of normal, adult blood mononuclear cells supplemented with GM-CSF plus IL-4. On day 2.5, small adherent DC aggregates are readily visible under the inverted phase contrast microscope (*A*). On day 7, the DC aggregates have become nonadherent, very large, and loose (*B*). The nonadherent fraction of the cultures was harvested and vigorously resuspended to obtain single DCs in large numbers (*C*, arrows mark some veils). (*A* and *B*) ×25; (*C*) ×500.

1 d, gently removed the nonadherent lymphocytes. We then observed the adherent cells every 12 h under the inverted microscope. To our surprise, many small adherent aggregates developed within 2 d, and most were covered with typical DC veils. However within two more days, the aggregated cells became round and gave rise to a monolayer of macrophages. These events took place whether GM-CSF or GM-CSF plus TNF were added. However, by day 12–16, typical expanding DC aggregates appeared in some of the wells. These aggregates were loosely affixed to an adherent monolayer as previously observed in mouse blood (11) (data not shown). The DCs that were released were typical in morphology, phenotype (data not shown), and T cell stimulatory function (Fig. 2 *C*). The yield was about 4% of the initial number of mononuclear cells plated, which is far greater than the 0.5–1% yield of DCs in fresh blood (28, 33).

We suspected from these findings that DC precursors were actually quite numerous in blood, but that the precursor still had the potential to give rise to macrophages. The latter is known to be the case for the CFUs that GM-CSF induces in mouse (34). Since IL-4 at 500–1,000 U/ml blocks macrophage colony formation (35), we added IL-4 to GM-CSF and repeated the experiments.

The combination of GM-CSF and IL-4 produced two striking findings. First, the numerous initial veiled aggregates (Fig. 3 *A*) did not transform into macrophages but rather increased rapidly in size over the next few days (Fig. 3 *B*). The aggregates became nonadherent, displayed typical veils all over the periphery, and began to release mature DCs (Fig. 3 *C*). Second, the single adherent cells (presumably monocytes) that were scattered in between the small adherent aggregates, also became nonadherent and developed processes similar to those of typical DCs (data not shown). Growing DC aggregates only formed in the presence of both GM-CSF and IL-4. The initial nonadherent fraction also developed some aggregates but these were obscured by the excess of lymphocytes.

After having made these observations in 20 experiments, we found it simpler to use larger 35-mm wells. The protocol was to plate $5–20 \times 10^6$ plain bulk mononuclear cells in 3 ml of medium, to discard the nonadherent cells at 2 h with a very gentle rinse, and to then culture the adherent cells in medium supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml). With the above gentle wash, the nonadherent cells did not develop DC aggregates, but with more vigorous washing, the aggregates mainly developed in the nonadherent fraction.

The presumptive DC aggregates were verified to be proliferating by two criteria: staining of ~10% of the cells with the Ki-67 mAb that identifies an antigen in cycling cells (27) (Fig. 4 *D*), and sensitivity to 3,000 rad. In contrast, the tightly adherent populations, which could develop single cells with the appearance of DCs (see above), were nonproliferating as evidenced by a lack of staining with anti-Ki-67 mAb (Fig. 4 *D*) and a resistance to 3,000 rad of irradiation.

The combination of GM-CSF and IL-4 reproducibly gives rise to large growing DC aggregates over a 5–7-d period.

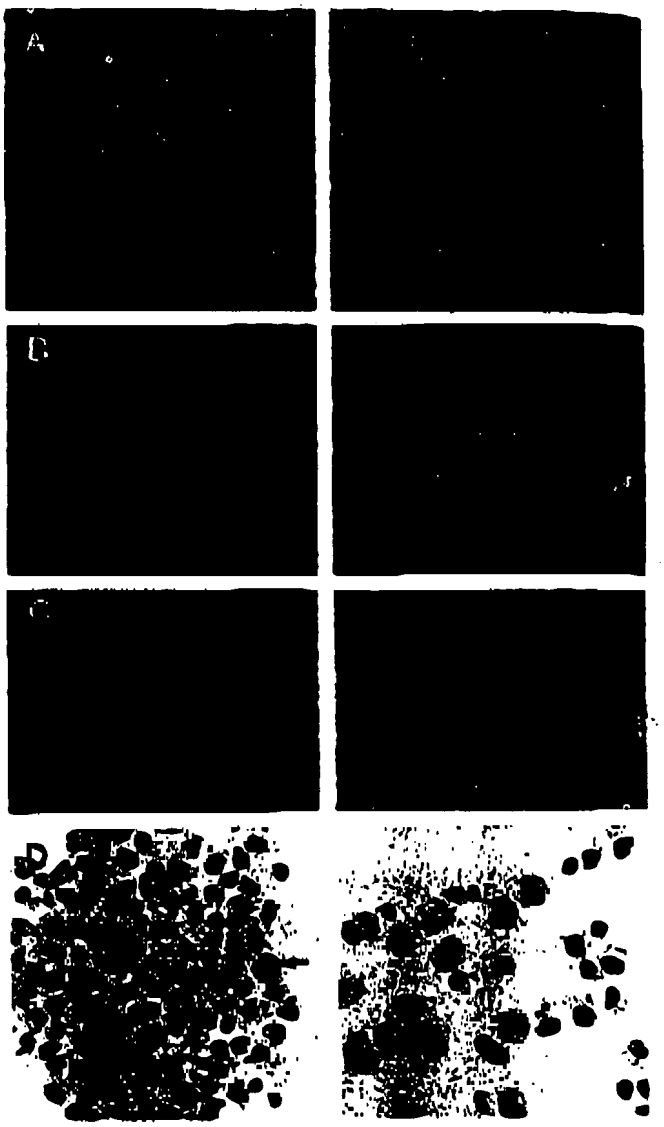

Figure 4. Phenotype and proliferation characteristics of DCs grown from normal blood with GM-CSF plus IL-4. Fluorescence pictures in each row represent identical microscopic fields of double-labeled cytospin preparations. (*Left*) Panels are stained with anti-HLA-DR. DCs grown in GM-CSF and IL-4 are strongly HLA-DR positive (*A*, *left*) but display only a dull spot of anti-CD68 reactivity (*right*). In contrast, control cells grown in parallel without cytokines (mainly macrophages) show an inverted pattern: very low HLA-DR (*B*, *left*) but brilliant CD68 expression (*right*). mAb Leu (*C*, *right*) identifies occasional Birbeck granule containing cells in the center of an HLA-DR-expressing aggregate of DC (*left*). Pseudo-color staining of nuclei and nucleoli with mAb Ki-67 (*D*) demonstrates that proliferation occurs predominantly in aggregates (*left*); singly dispersed DC derived from firmly adherent cells (see text) are not stained (*right*). (*A–C*) ×200; (*D*) ×100.

At that time, growth essentially ceased. The aggregates then could be disassembled by pipetting into DCs with a typical surface phenotype (Fig. 5), characteristic morphology at the light (Fig. 3 *C*) and electron microscopy levels (see Fig. 6), and strong T cell stimulatory function (Fig. 2, *D–F*). Human rIL-1 (50 LAF U/ml), when added during the last 24 h of culture, amplified the stimulatory function of DCs as ob-

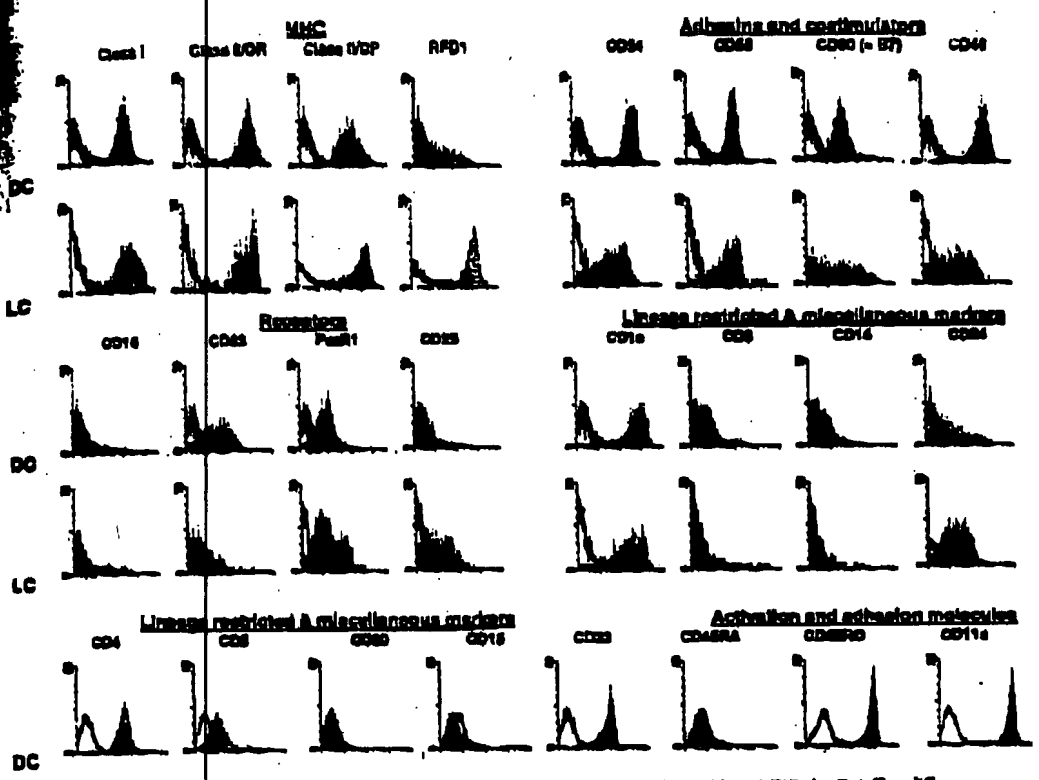

Figure 3. Cytofluorographic analysis of dendritic cells (*DC*) grown from normal peripheral blood with GM-CSF plus IL-4. Two different representative experiments are shown. Epidermal Langerhans cells (*LC*) cultured for 3 d were included in one experiment for comparison. Three-color immunolabeling was performed. Cells were stained with different mouse mAbs followed in sequence by biotinylated anti-mouse Ig, streptavidin-PE, mouse Ig for blocking free binding sites, and FITC-conjugated anti-HLA-DR. Dead cells and lymphocytes were excluded from analysis by propidium iodide staining and light scatter properties, respectively. More than 90% of the remaining cells were strongly MHC-class II positive and constituted DC. The phenotype of this population is shown (*shaded curves*). Isotype-matched control antibodies are included in each histogram (*bold curves*). Blood DC display a phenotype typical for DCs as described and almost identical to cultured LC in direct comparison (24, 28, 29, 33). It is notable that they do not express CD14 but have high levels of MHC molecules (HLA-ABC, DR, DQ, and DP), adhesins (CD54, CD58, CD11a [not shown], and CD11c), and costimulatory molecules (CD40 and B7/CD80). They are also negative with markers for granulocytes (CD15), NK cells (CD16), B cells (CD19 [not shown] and CD20), and T cells (CD3 and CD8 [not shown]). Expression of CD3 and the staining pattern of CD45RA and -RO are as described for DCs isolated from fresh blood (33).

served with murine DCs isolated from spleen or epidermis (18, 36). It is interesting to note that the blood-derived DCs expressed CD1a, CD4, and FcεRI as is typical of epidermal Langerhans cells (25, 37, 38). Birbeck granules were not detectable by electron microscopy, however, and only a rare cell in the center of a residual DC aggregate stained with anti-Lag mAb (26) (Fig. 4 *C*). Anti-CD68 immunostaining revealed a perinuclear zone of reactivity in some of the DCs (Fig. 4 *A*), a feature that differs from the strong diffuse granular staining of macrophages (Fig. 4 *B*).

The yield of mature, immunostimulatory DCs (Table 1) was 6–15% of the mononuclear cells plated. This is many times greater than the number of DCs that can be identified in unstimulated blood (0.3–1%) (28, 33). The above protocol and yield (3–8 × 10^6 DCs/40 ml of blood) has proven reproducible in over 25 experiments with blood from healthy males and females (25–60-yr-old), using either fresh venipuncture or buffy coat preparations.

Discussion

DC Progenitors in Human Blood: Identification. These findings of necessity appear methodological in nature but in fact outline a pathway whereby the distinct DC lineage can be induced to proliferate and mature from precursors that are relatively plentiful in human blood. The methodological caste of our results reflects the difficulty inherent in identifying precursors and progeny in this distinctive immunostimulatory pathway. DCs are not yet known to express a lineage-specific surface antigen, as is the case with lymphocytes, e.g.,

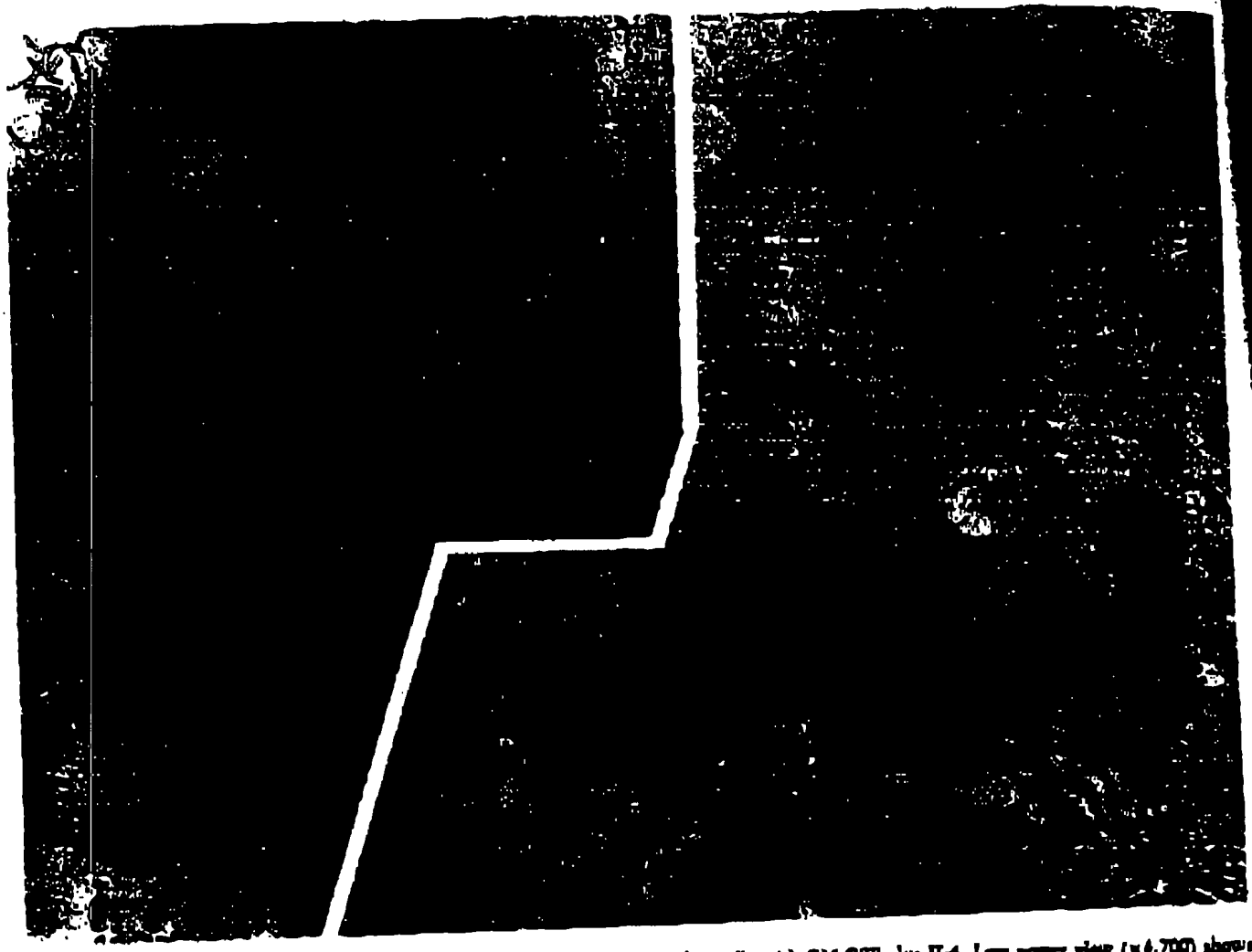

Figure 6. Ultrastructure of DCs grown from normal, adult blood monocuclear cells with GM-CSF plus IL-4. Low power view (×4,700) shows three profiles of DCs (*Arrowheads*) Veils, i.e., thin cytoplasmic processes devoid of organelles. (*Border*) Area shown at higher magnification (×33,000) to demonstrate the characteristic abundance of mitochondria and paucity of lysosomes/phagosomes.

CD3, CD19, and CD20. A lack of lineage-specific markers is also typical of the individual human myeloid lineages, e.g., monocytes, neutrophils, basophils, and eosinophils. However, these other myeloid lineages have distinctive functional properties and distinctive CSFs, e.g., M-CSF and G-CSF. DCs, in contrast, are only known to respond to the multilineage cytokine GM-CSF (17, 18, 39), and their peculiar morphology, phenotype, and function is best outlined with a composite of approaches (1).

Given these inherent difficulties, we searched for criteria that were similar to those that had been used to identify immature DC progenitors (e.g., MHC class II negative) in mouse blood (11) and bone marrow (12). Mouse DCs proliferate within a characteristic aggregate that attaches loosely to an underlying stroma and is covered with large sheetlike processes or veils (compare Figs. 1 and 3). By defining conditions that give rise to such aggregates, at first containing a few cells but growing to >10 cells in diameter, we could establish that proliferating DC progenitors are readily detectable in the blood of all healthy adults, and that one could use these progenitors to generate relatively large numbers of typical immunostimulatory DCs within 7 d, i.e., 3–8 million of such cells per 40 ml of blood.

The critical finding was that GM-CSF did induce the formation of many small DC progenitor aggregates in human blood, but that these did not proliferate further and seemed to become typical macrophages. IL-4, a known inhibitor of macrophage colony formation (35), allowed extensive DC growth and maturation to ensue (Fig. 3).

DC Progenitors in Human Blood: Cytokine Requirements. To study the properties of DC progenitors in blood, it is not necessary to enrich for $CD34^+$ multilineage progenitors which are so rare (<0.1%) in normal blood (40). The need for exogenous cytokines may vary from one experimental situation to another depending on their endogenous production (e.g., TNF) by cells in the culture. However, it is to

essential to add GM-CSF. Exogenous TNF-α is useful increase DC numbers and function, as described by Caux [14], but primarily when one uses cord blood from who are receiving CSF therapy to compensate for therapy. The function of TNF-α may be to diminish locyte production (41, 42), and to enhance responsive- of an early progenitor to GM-CSF as by inducing the chain of the GM-CSF receptor (41, 42). With normal adult blood, IL-4 is the desired exogenous cytokine that is to be added in combination with GM-CSF. We suspect that IL-4 acts by suppressing the monocyte differentiation potential of the DC progenitor (35).

GM-CSF is essential to grow DCs from all sources used. Additional cytokines required for optimal DC growth from the various sources are, however, strikingly different (TNF-α versus IL-4). We suspect that this is due to the fact that the main DC progenitors involved differ. In cord blood the DC aggregates likely derive from CD34$^+$ cells as preliminary experiments (Romani, N., unpublished observations) have shown that depletion of CD34$^+$ cells from the initial inoculum virtually abolishes the formation of DC aggregates. This also readily explains the need to add TNF-α which is known to induce responsiveness to GM-CSF of CD34$^+$ cells (41, 42). Ongoing experiments indicate that IL-4 does not seem to enhance DC development from precursors that arise in cord blood mononuclear cells supplemented with GM-CSF and TNF-α (Brang, D., unpublished observations). We do not yet know, however, whether IL-4 is produced endogenously in such cultures. Endogenous IL-4 might suppress—similar to exogenously added IL-4 in adult blood cultures—the monocyte differentiation potential of more mature DC progenitors that derive from CD34$^+$ multilineage progenitors in response to GM-CSF and TNF-α. DC developmental pathways in cultures of blood derived from cancer patients during hematopoietic recovery are presumably similar to cord blood. Besides CD34$^+$ cells it is, however, likely that more committed precursors are also involved as the percentage of CD34$^+$ cells in the CD3/HLA-DR–depleted mononuclear cell fraction did not strictly correlate with DC yields. In normal adult blood in response to GM-CSF and TNF-α and only after a prolonged culture period (2 wk), some DC aggregates emerged probably from early, rare DC progenitors similar to those in cord blood or the blood of cancer patients during hematopoietic recovery. The main DC progenitor(s) in normal adult blood, however, appear(s) to be more frequent as only 2 d of culture are needed before many DC aggregates appear (Fig. 3). Prior work in mice (34) and in humans (16) has described that the multilineage colonies that are induced by GM-CSF in semisolid agar cultures contain all three types of myeloid progeny, i.e., granulocytes, macrophages, and DCs. The principal DC progenitor in normal human peripheral blood seems more differentiated since granulocytes do not develop. This committed progenitor is GM-CSF responsive, and likely bipotential, developing into macrophages rather than DCs unless its monocyte differentiation potential is suppressed by IL-4.

DC Progenitors in Human Blood: Clinical Relevance. The larger numbers of DCs that are now available should help characterize two other intriguing features of the DC phenotype that are clear-cut in the progeny that can be reared with GM-CSF and IL-4 from human blood. One is the expression of FcεRI receptors which could have a role in atopic dermatitis (25, 38), e.g., via the presentation of small amounts of antigen as IgE complexes. A second feature is the high level of expression of CD4, the principal receptor for HIV-1 (43).

It also may be worthwhile to consider the use of IL-4 and GM-CSF, perhaps together with antigen-pulsed DC progenitors (22), to enhance immune responses in situ. In any case, methods for the identification and growth of DC progenitors in human blood, especially normal adult human blood, should make it feasible to explore the immunogenic potential of these cells in clinical situations, such as the presentation of antigens in resistance to infections and tumors.

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